FEEDING THE WORLD IN THE 21ST CENTURY: GRAND CHALLENGES IN THE NITROGEN CYCLE

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Executive Summary

The purpose of this workshop, held November 9–10, 2015 at the National Science Foundation headquarters in Arlington, Virginia, was to identify ways that chemists can help the scientific community understand and manage the nitrogen cycle to improve agriculture and environmental quality. The workshop was convened by Principal Investigator Nicolai Lehnert of the University of Michigan and co-investigators Gloria Coruzzi of New York University, Eric Hegg of Michigan State University, Lance Seefeldt of Utah State University, and Lisa Stein of the University of Alberta. Participants included chemists and biologists from a wide range of U.S. and international institutions and a wide range of subdisciplines within their respective fields. (See Appendix 1 for a full list of participants.)

Participants noted two major imbalances in the nitrogen cycle: Not enough nitrogen is available to plants growing in soils in the developing world, and too much inorganic nitrogen is often available in soils in the developed world. Addressing the first imbalance requires that scientists (1) better understand and find ways to control nitrogen fixation, which converts atmospheric dinitrogen (N_2) into bio-accessible forms like ammonia (NH_3/NH_4^+) and nitrogen oxides, and/or (2) improve nitrogen assimilation into plants, so that plants and, ultimately, people gain access to more of the nitrogen in soil.

Addressing the second imbalance requires that scientists better understand and find ways to control the other processes in the nitrogen cycle: nitrification (the biological oxidation of nitrogen in soil), denitrification (the biological reduction of nitrogen in soil), and, again, nitrogen assimilation into plants.

On the workshop's first day, participants were divided into four groups based on different, though interrelated, processes or topic areas within the nitrogen cycle: nitrogen fixation, nitrification, denitrification, and nitrogen assimilation. Participants were encouraged to identify big questions and brainstorm blue-sky research approaches to their respective topics, without regard to resource or technological constraints.

On the second day, workshop participants regrouped in interdisciplinary teams focused around specific questions:

- How does reduction of N_2 to NH_3 work at an atomic level in the nitrogenase enzymes and in homogeneous and heterogeneous artificial (man-made) catalysts?
- How can we breed or engineer biological nitrogen fixation capabilities into a wider range of organisms?

- How can we effectively manage nitrification at an ecosystem level?
- How can we maximize nitrate reduction while simultaneously limiting nitrous oxide production and subsequent release into the atmosphere during denitrification?
- What are the mechanisms of enzymes that catalyze nitrogen interconversions and chemical bond formation between inorganic nitrogen and either carbon (amination) or sulfur (nitrosylation) in microbes and plants?
- What chemical features of nitrogen-based signaling molecules regulate changes in nitrogen assimilation in plants?
- How can we promote environmentally resilient nitrogen use efficiency, especially in anticipation of changing environmental factors?

Within each of the four topic areas, participants identified big questions, knowledge gaps, expertise required, experimental approaches, technology needed to make progress, and specific desired outcomes. The key recommendations that emerged from this workshop are:

- Develop a better understanding of the atomic-level mechanism of all three catalyst types involved in nitrogen fixation (enzymatic; homogeneous and heterogeneous artificial).
- Advance the use of electrocatalysts (catalysts that function at electrode surfaces) for nitrogen fixation.
- Seek currently unknown nitrogen fixation systems in archaea, prokaryotes, and eukaryotes.
- Develop a full understanding of the expression levels and timing of all nif (nitrogen fixing) genes, to enable maximum biological nitrogen fixation activity.
- Discover and develop comprehensive, specific, environmentally benign, and cost-effective nitrification inhibitors using directed approaches.
- Develop large-scale, predictive ecosystem nitrogen flux models for data-driven application of fertilizer/inhibitor formulations, to monitor nitrogen flux in complex microbial communities, and to measure nitrogen exchange between plants and their environment.
- Study a greater diversity of organisms that reduce nitrate, and better understand which of these organisms is dominant under different growth conditions.

- Identify strategies to alter the regulation of the nitrate reduction pathways, in order to control the flux through the various pathways (and especially to enhance nitrate reduction to ammonia).
- Ascertain the detailed molecular mechanisms of the key enzymes involved in nitrate reduction (denitrification and nitrate reduction to ammonia) and ammonia oxidation (nitrification) pathways.
- Integrate knowledge about regulatory networks controlling nitrogen assimilation, responses to other plants stresses, and plant growth into a systems-level understanding of how nitrogen powers plant productivity.
- Develop crop breeding or genetic engineering methods to rapidly reprogram plants' gene regulatory networks to optimize the use of the relatively large and uniform nitrogen supplies available in managed cropping systems.

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Introduction

The nitrogen cycle is one of the most important biogeochemical cycles on Earth, as nitrogen is an essential nutrient for all known life forms. Nitrogen is abundant in the air in the form of gaseous dinitrogen (N_2), but for plants to use it, it must first be "fixed," or converted into other forms, such as ammonia (NH_3/NH_4^+) and various nitrogen oxides.[1]

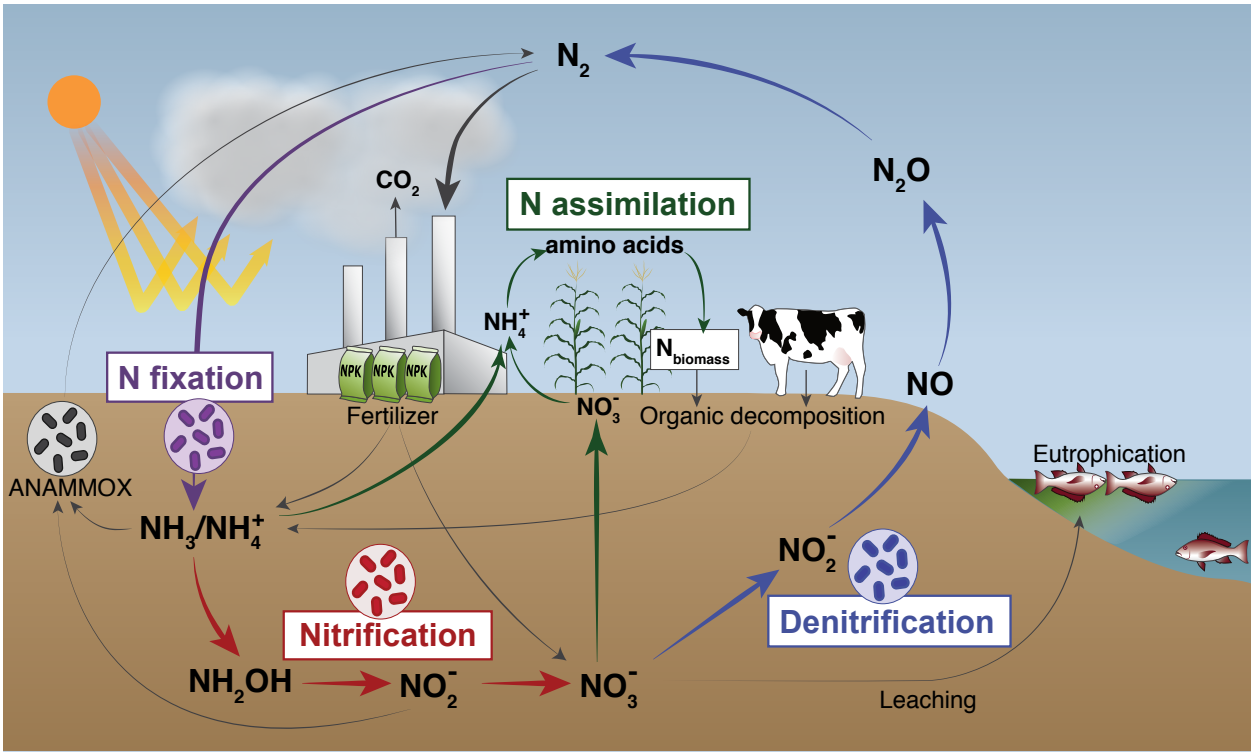


Figure 1. *The main processes in the nitrogen cycle.*

Natural processes, driven mostly by microbes in association with leguminous plants, fix and deliver around 120 megatons per year of bioavailable nitrogen to the biosphere (Smil, 2001). Humans have greatly augmented these processes, mostly through the

1 Please note that ammonia (NH_3) undergoes an acid-base equilibrium in water, $NH_3 + H_2O \leftrightharpoons NH_4^+ + OH^-$. At the pH typical in soil and ground or river water, ammonia is found exclusively as ammonium (NH_4^+) ions. For this reason, whenever we refer to ammonia in the environment, it is denoted as NH_4^+.

industrial Haber-Bosch process, which uses a solid-state, iron-based catalyst to mediate the reaction

$$N_2 + 3\ H_2 \rightarrow 2\ NH_3 \quad (1)$$

and through planting of leguminous crops such as soy, alfalfa, beans, and peas.

These processes contribute at least the same amount of fixed nitrogen as do natural processes. Experts have estimated that around 40% of the human population depends on the human contribution to the nitrogen cycle (Smil, 2001; Erisman et al., 2008).

Because soils in the developing world often lack bioavailable nitrogen, crop yields are often low and diets are often lacking in protein. To address this challenge, scientists must find ways to increase the amount of nitrogen availability and/or how much nitrogen plants take up. Because transporting nitrogen fertilizer is often costly and inefficient, solutions may involve developing new crops or other technologies that can fix nitrogen in the field.

In the developed world, nitrogen-containing fertilizer is cheap enough that farmers routinely add enough to fields to ensure that nitrogen is never a limiting nutrient. On average, only 30% to 50% of this nitrogen is taken up, or assimilated, by crop plants. The rest is converted between various forms by soil microbes. These interconversions generally take the form of nitrification, which is oxidation from ammonium to nitrite (NO_2^-) and nitrate (NO_3^-); or denitrification, which is reduction through nitric oxide (NO) and nitrous oxide (N_2O) back to N_2. Dissimilatory reduction of NO_3^- to NH_4^+ is another microbial process, although scientists are unsure how much this process contributes to total nitrogen cycling in soils.

Nitrification and denitrification can lead to multiple environmental problems, because many of the chemical products of these processes readily leach from soil into water or escape into the atmosphere. Two of the most severe problems are nitrate runoff into waterways, which causes downstream eutrophication and "dead zones"; and release of N_2O, a potent greenhouse gas, and NO, an ozone depleting gas (Galloway, 2013).

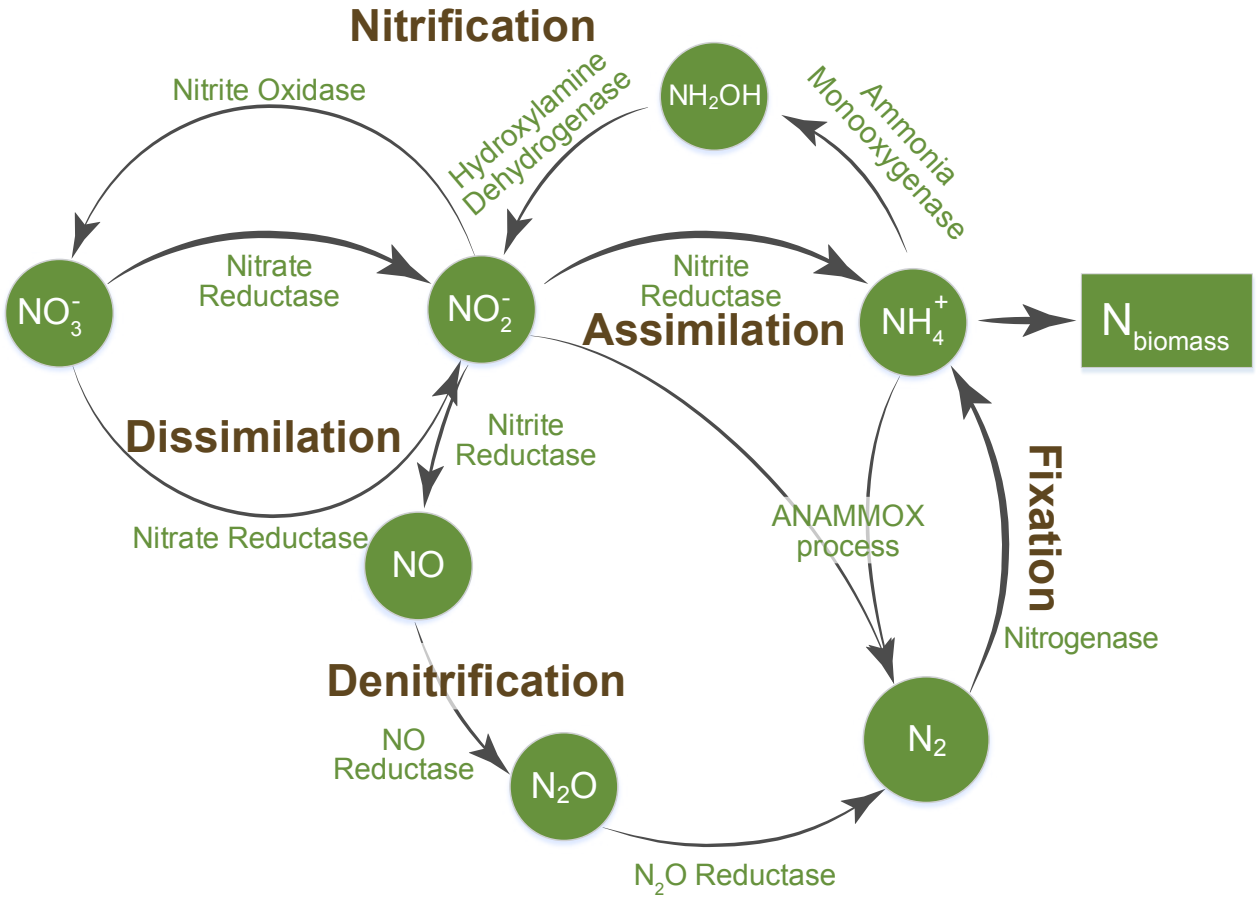


Figure 2. *Enzymes involved in the nitrogen cycle.*

Grand challenges identified by workshop participants involve reducing these environmental impacts by better matching fertilizer applications to crop needs, creating nitrogen flux models to aid in this, developing new nitrification inhibitors, developing methods to remove nitrates from soil to limit runoff, inhibiting the production of nitrous oxide in soil, and increasing how much nitrogen plants take up.

Improving the bioavailability of nitrogen

Question 1: How does reduction of N_2 to NH_3 work at an atomic level in the nitrogenase enzyme and in homogeneous and heterogeneous artificial catalysts?

Background

The reduction of nitrogen (N_2) from the atmosphere to ammonia (NH_3/NH_4^+) is called nitrogen fixation. More nitrogen enters the global biogeochemical nitrogen cycle in this manner than through any other means. Currently, two processes, roughly equal in magnitude, account for the majority of nitrogen fixation. These are (1) biological nitrogen fixation in bacteria catalyzed by enzymes called nitrogenases, and (2) the industrial Haber-Bosch process (Erisman et al., 2008).

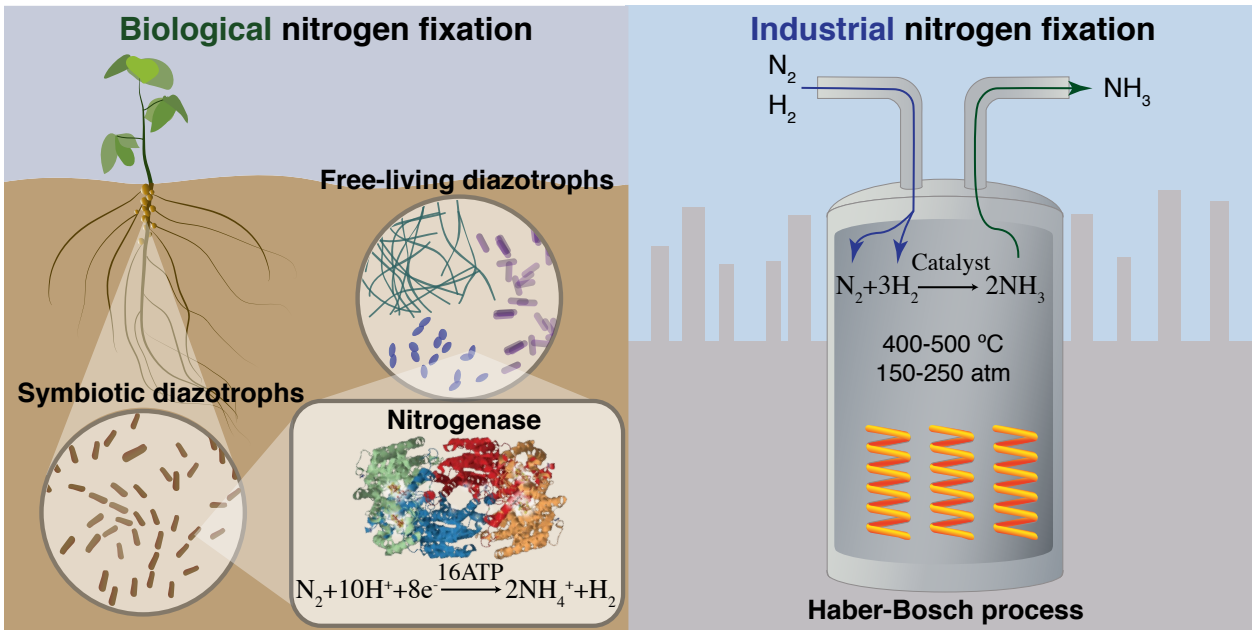


Figure 3. *Biological (left) and industrial (right) processes fix roughly equal amounts of nitrogen (Protein structure from Spatzal et al., 2011).*

These two processes use very different conditions and mechanisms to reduce N_2 (see Figure 3). Biological nitrogen fixation relies on a set of bacteria and archaea collectively called diazotrophs. Diazotrophs are best known for forming associations with the roots of leguminous plants and producing ammonia for the plant in exchange for sugars;

however, there are also diazotrophs that form associations with non-leguminous plants, and even with fungi and animals. Others are free-living. The key enzymes that catalyze N_2 reduction in diazotrophs are called nitrogenases (Burgess and Lowe, 1996; Eady, 1996).

The industrial Haber-Bosch process, by contrast, uses iron-based catalysts at 150 to 250 times atmospheric pressure and between 400 and 500 °C. Though the process is more efficient than any other known for converting N_2 to NH_3, it still relies heavily on fossil fuels both to provide hydrogen and to achieve (through combustion) the high temperatures and pressures needed for the reaction.

Roughly 100 million tons of nitrogen fertilizer are produced each year via the Haber-Bosch process, which is responsible for around 3% of global fossil fuel consumption (Smil, 2011). Haber-Bosch chemistry has been a great success, but it will be increasingly challenging to sustain it in its current form as fossil fuel reserves dwindle.

Gaining a molecular-level understanding of both natural and industrial nitrogen fixation processes is a key foundation of research going forward. We need to better understand how nitrogenases activate and reduce N_2 (Hoffman et al., 2014). And we need to develop new homogeneous and heterogeneous catalysts such as those used in the Haber-Bosch process[2].

This foundation will help us both achieve nitrogen fixation chemistry under milder conditions using less fossil fuel than is currently achievable, and build distributed systems that allow ammonia production from N_2 to be decentralized, which is crucial in developing countries where transportation of fertilizer can be difficult or impossible. In addition, electrocatalytic N_2 fixation eliminates the need for H_2, and such technology could be deployed in rural areas for solar-driven ammonia production on a small scale.

Significant knowledge gaps remain in all of these areas, however, and we need coordinated research to fill these gaps.

Research Approaches and Technology Needed

- Develop a better understanding of the atomic-level mechanism of all three catalyst types (enzymatic, homogeneous artificial, and heterogeneous artificial). To do this, researchers will need to employ a wide range of spectroscopic and structural technologies, with the goal of atomic-level analysis of the catalytic sites.

2 Heterogeneous catalysts have different phases than the reactants in a chemical reaction, whereas homogeneous catalysts have the same phase as the reactants.

Ultimately this approach will provide foundational knowledge that can lead to the development of new catalysts.

- Deduce key intermediates along the reaction pathway that nitrogenases or other catalysts use to reduce N_2 to NH_3. Once we know these intermediates, we can construct a reaction pathway, which in turn will provide insights that can be extended to novel catalyst designs. To characterize these intermediates, scientists will have to trap enzymes or synthetic catalysts "in action" and study the trapped states by different methods such as spectroscopy and electrochemistry.
- Understand how to achieve N_2 reduction without using H_2, the production of which currently relies on natural gas. This approach could be based on electrocatalysts, which are catalysts that function at electrode surfaces. Such catalysts could provide electrons and protons from an abundant source, such as water. Combined with photovoltaics, this technology could be used for the decentralized, solar-driven production of ammonia from N_2 on a smaller scale.
- Use computational modeling to extend and fill in mechanistic knowledge gaps. Modeling should be grounded in experimental observations and calibrated with experimental data.
- Extend the effectiveness of Haber-Bosch chemistry, while pursuing new approaches using modern materials (e.g., metal organic frameworks and thin-film electrodes). Such materials could allow the process to run at lower temperatures, reducing the energy needed to drive the process.

Expertise Needed

The key to success will be organizing teams of chemists, biochemists, and engineers to work on common challenges. Organized research teams operated in the 1980s at the Nitrogen Fixation Laboratory in Sussex, UK and the Kettering Laboratory in Yellow Springs, Ohio. These teams made significant strides in understanding nitrogen fixation. There is once again a need for organizing cross-disciplinary teams to move the field forward.

Outcomes

The research program outlined above will produce fundamental knowledge that will help produce low-temperature, low-energy, robust catalysts that can be distributed at low cost and that can be used to fix N_2 electrocatalytically for the local production of ammonia.

Question 2: How can we breed or engineer biological nitrogen fixation capabilities into a wider range of organisms?

Background

We need to better understand the steps involved in creating active nitrogenases, to allow us to deploy this process in species of interest, e.g., plants (Oldroyd and Dixon, 2014; Rogers and Oldroyd, 2014). This will involve creating new symbiotic relationships between plants and nitrogen-fixing bacteria as well as engineering nitrogen fixation genes directly into plants and perhaps other life forms.

Tremendous knowledge gaps remain in many core areas, including understanding how to mobilize all the genes needed for nitrogen fixation into new prokaryotes and eukaryotes, via both traditional breeding and genetic engineering (Curatti and Rubio, 2014; Geddes et al., 2015). Furthermore, we must explore the full range of nitrogen-fixing organisms to better understand the range of approaches used by biology (Gresshoff et al., 2015; Thomas et al., 2015). Currently most research done on biological nitrogen fixation focuses on bacteria that form associations with a limited set of leguminous plants, most of which are food or forage crops.

Research Approaches and Technology Needed

- Seek currently unknown nitrogen fixation systems in archaea, prokaryotes, and eukaryotes.
- Use synthetic biology to create new nitrogen fixation systems beyond those that currently exist in the biological world.
- Develop the ability to measure nitrogen fixation in the field with minimal invasion of the plant.
- Develop a full understanding of the expression levels and timing of all of the nif (nitrogen fixing) genes to enable maximum biological nitrogen fixation activity.
- Develop a means for moving nitrogen fixation into eukaryotes via genetic engineering.

Expertise Needed

Best progress will be made by assembling a team that includes biologists, chemists, biochemists, systems biologists, synthetic biologists, and ecologists. Building teams with knowledge across nitrogen fixation and eukaryotic genetics will be valuable.

Outcomes

The research program outlined above will produce fundamental knowledge of how biological nitrogen fixation works, which will help scientists deploy and control nitrogen fixation across prokaryotes and eukaryotes via both traditional breeding and genetic engineering.

Controlling nitrogen interconversion in soil, increasing nitrogen available to plants, and suppressing environmental impacts

Question 1: How can we effectively manage nitrification at an ecosystem level?

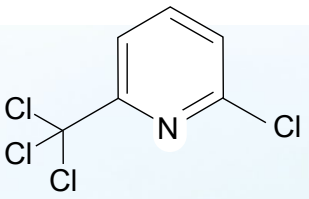
Background

Nitrification, the oxidation of ammonium to nitrate, is the main process that connects biologically reactive nitrogen to other processes in the nitrogen cycle (Stein, 2016; Ward, 2011). Specifically, nitrification provides nitrate (and nitrite) to denitrifying bacteria in the soil. Microbial nitrification is also the main process that competes with plants for applied anhydrous ammonium fertilizer, and that determines how much ammonium and nitrate are available for assimilation into plants.

Beyond its importance for agriculture, nitrification has serious environmental implications. Nitrogen oxide intermediates NO and N_2O are gases, and can leak out of the soil and into the atmosphere, resulting in ozone depletion and increased greenhouse effect (Galloway et al., 2013). Nitrate and nitrite also easily leak out of soil into waterways. From there they make their way to lakes and oceans, where they fertilize algae and cyanobacteria blooms. Ultimately this leads to eutrophication and low-oxygen zones ("dead zones"), such as the one that appears every year in the Gulf of Mexico.

There are a tremendous number of open questions in nitrification. Although the first ammonia- and nitrite-oxidizing bacteria were isolated more than 125 years ago, the enzymology and physiology of these microbes remain largely mysterious. One of the principal enzymes involved in ammonia oxidation, ammonia monooxygenase, has defied purification, preventing scientists from understanding its active site structure and how it works.

Nitrapyrin
nitrification inhibitor



Scientists have sought nitrification inhibitors that would minimize the environmental problems described above. Nitrapyrin (see previous page) is the main inhibitor applied to agricultural soils in the U.S. today, and has been widely used since 1974. Even so, scientists do not understand how nitrapyrin works, and it is not even known to be specific to ammonia oxidizers; it might target nitrite oxidizers, methane oxidizers (as they also express a monooxygenase enzyme), or multiple groups of microorganisms (Fisk et al., 2015).

Figure 4. *Satellite image of Great Lakes cyanobacteria blooms caused by agricultural runoff. Credit: NASA*

In addition, the recent discovery of ammonia-oxidizing archaea and "comammox" bacteria (Daims et al., 2015) that oxidize ammonia all the way to nitrate greatly expanded the realm of possible enzymology involved in the nitrification process. Because of this enzymatic and organismal diversity, scientists do not yet know of a comprehensive and specific nitrification inhibitor.

To effectively manage nitrification, workshop participants proposed a two-pronged approach: (1) discover and develop comprehensive, specific, environmentally benign, and cost-effective nitrification inhibitors using directed research approaches; and (2) develop large-scale, predictive ecosystem nitrogen flux computer models to predict plant and soil nitrogen status for data-driven application of fertilizer/inhibitor formulations.

For these models, researchers will need to collect experimental data with sensors (which could be deployable sensors or plants), and feed them into mathematical models to predict how nitrogen moves through the soil. The models will be useful for all areas of the nitrogen cycle, including nitrogen fixation, nitrification, denitrification, and assimilation. The success of this approach requires an interdisciplinary combination of expertise and methodologies, and also relies on engagement with policy makers and industry.

Research Approaches and Technology Needed

To develop specific, targeted nitrification inhibitors:

- Examine a range of existing nitrification inhibitors and their modes of action, and explore mechanisms of inhibition using synthetic models. Ultimately the goal is to create novel inhibitors through combinatorial chemistry.
    - » Isolate, express, and purify the key enzymes involved in nitrification from different organisms to explore the biodiversity of these enzymes, and determine the mechanisms of these enzymes on a molecular level, using kinetic, spectroscopic, theoretical, and model studies. This will require access to enzymes via overexpression technologies and purification, as well as single-molecule and single-cell technologies. High-resolution cryoelectron microscopy can be used to obtain molecular structures. Selective inhibitors can also be employed to characterize enzymes and the diversity of catalytic mechanisms.
    - » Develop suitable synthetic (chemical) model systems for the active sites of the enzymes to test mechanistic hypotheses and explore the basic chemistry that these sites could mediate.
- Assess the effects of nitrification inhibitors across functional groups of nitrifying microorganisms.

- Explore structure/function relationships of inhibitors across homologous enzymes, design inhibitors based on enzyme structure, determine the specific targets of inhibitors in enzymes of nitrification, and synthesize the targeted compounds.
- Elucidate factors that facilitate coupling and uncoupling of ammonia to nitrite oxidation as well as factors that govern the leakage of intermediate molecules during nitrification.
- Determine the environmental fate of nitrification inhibitors, and the influence of changing environments on communities and activities of nitrifying organisms.
    - » Find more effective and environmentally friendly nitrification inhibitors; screen for new natural compounds in small-scale, laboratory-based studies; and identify natural inhibitors and their genes from plants or other previously identified and publicly available sources. This will require access to natural product libraries from industry.
    - » At the plant level, study signaling between plants and nitrifying microbes to determine what triggers the production of inhibitors, and characterize how plants synthesize and regulate production of natural nitrification inhibitors.
    - » Develop a better understanding of the organismal and genomic diversity involved in nitrification.
    - » Develop more sophisticated fertilizer formulations and applications, so that results from this research can be used in agriculture. Develop an understanding of how global change and local changes influence soil nitrogen levels that in turn influence applications of fertilizer and inhibitors.

To develop better ecosystem nitrogen flux models:

- Use plants (bred or engineered) as sensors for nitrogen availability in soil; optimize or create new chemical, physical, and probe soil sensors; and correlate plant and soil sensor systems.
- Develop solid-state sensors for NO_3^-, N_2O, and NH_4^+ that work in soils and are simple, inexpensive, sensitive, and fast. (This could include electrochemical approaches.)
- Develop predictive nitrogen flux models, iteratively test them, and correlate the results to soil type and cropping systems.

» Develop advanced nutrient monitoring abilities, e.g. of nitrogen fixation, using large-scale sensor networks for nitrogen status and robust sensor technology for nitrogen and related nutrients.

Expertise Needed

Progress will require collaboration between chemists with a variety of subspecialties as well as experts from outside chemistry. Within chemistry, expertise is required in biochemistry, bioinorganic and synthetic chemistry, analytical chemistry and engineering, computational chemistry, and biogeochemistry. Additional expertise is needed from plant biology and plant breeding, chemical and microbial ecology, microbial physiology, molecular genetics, and biophysics.

Outcomes

We hope that research on specific, targeted nitrification inhibitors combined with molecular-level mechanistic studies of key nitrifying enzymes will enable us to find a "perfect" nitrification inhibitor that is environmentally benign and inexpensive to produce and apply, and that leaves ammonium available for plant growth.

Research on ecosystem nitrogen flux models will help us understand the effects of nitrification inhibitors on soil systems, predict nitrogen status and nitrogen losses of crops and soils, monitor related nutrient status of crops and soils, ease deployment and data collection across a landscape, and monitor nitrogen fixation status.

Question 2: How can we maximize nitrate reduction while simultaneously limiting nitrous oxide release during denitrification?

Background

As mentioned above, nitrate (NO_3^-) produced via nitrification can readily leach from soils and cause serious environmental problems. Massive "dead zones" now occur annually in many of the world's major water bodies due to the accumulated effects of nitrate (as well as phosphate) runoff, causing cyanobacteria and algae blooms that can deoxygenate large volumes of water. The Gulf of Mexico dead zone resulting from nutrients in the Mississippi River can grow as large as the state of New Jersey. Blooms of certain species can also be toxic and sicken animals living or swimming in water bodies, and can in some cases endanger human health, as happened in summer 2014, when a nitrate- and phosphate-fueled cyanobacteria bloom in Lake Erie made Toledo, Ohio's drinking water

unsafe. For both environmental quality and human health, it is critical to limit nitrate in waterways.

Denitrification is the process that reduces nitrate and nitrite to other compounds and ultimately to elemental nitrogen (N_2) or ammonia (NH_3/NH_4^+). There are two major mechanisms by which microbes use nitrogen oxide compounds as electron acceptors under anaerobic conditions. One is through the dissimilatory nitrate reduction to ammonia (DNRA) pathway, in which nitrate is reduced to nitrite and then further reduced by six electrons to ammonia (Rutting et al., 2011; Simon, 2002; Tiedje, 1988). The equations for conversion of nitrate to ammonia via the DNRA pathway are:

$$NO_3^- + 2\ e^- + 2\ H^+ \rightarrow NO_2^- + H_2O \quad (2)$$

$$NO_2^- + 6\ e^- + 8\ H^+ \rightarrow NH_4^+ + 2\ H_2O \quad (3)$$

The other major nitrate reduction pathway is denitrification, a multistep process by which NO_3^- is reduced to N_2 (Averill, 1996; Averill, 2007; Zumft, 1997). The steps for conversion of nitrate to nitrogen via denitrification, during which N_2O is produced in an intermediate step, are:

$$2\ NO_3^- + 4\ e^- + 4\ H^+ \rightarrow 2\ NO_2^- + 2\ H_2O \quad (4)$$

$$2\ NO_2^- + 2\ e^- + 4\ H^+ \rightarrow 2\ NO + 2\ H_2O \quad (5)$$

$$2\ NO + 2\ e^- + 2\ H^+ \rightarrow N_2O + H_2O \quad (6)$$

$$N_2O + 2\ e^- + 2\ H^+ \rightarrow N_2 + H_2O \quad (7)$$

Another way to represent this pathway is:

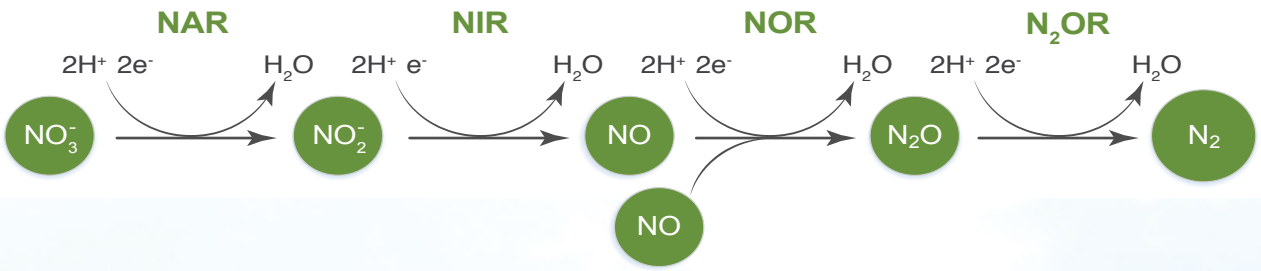


Figure 5. *Steps of the denitrification pathway (NAR, NIR, NOR, and N_2OR are abbreviations for the involved enzymes).*

Together, these two processes make up a critical aspect of the global nitrogen cycle. Denitrification is the more common pathway in most ecosystems, and has received a considerable amount of attention due to its impact on the environment. The final step in the denitrification pathway is the reduction of N_2O to N_2. Certain organisms, however, lack the enzyme required for this final step and instead release N_2O (Shoun et al., 2012).

In other instances, N_2O simply escapes to the atmosphere prior to reduction. In fact, around 75% of all anthropogenic N_2O emissions result from microbial activity in agricultural soil (U.S. EPA, 2016). Not only is N_2O a major contributor to the destruction of the ozone layer, it also has a 100-year greenhouse warming potential nearly 300 times higher than CO_2 (U.S. EPA, 2016). Globally, N_2O traps more radiation than any anthropogenic greenhouse gas other than carbon dioxide (CO_2) and methane (CH_4) (IPCC, 2007).

To mitigate these serious environmental problems, we need a better understanding of both the denitrification and the DNRA pathways, especially with respect to the diversity and physiology of microbes involved. In addition, we need to know more about the factors that regulate these pathways, including enzyme assembly and maturation processes, and about the key enzymatic reaction mechanisms. This knowledge will enable us to ultimately promote nitrate reduction via the DNRA pathway while simultaneously limiting nitrous oxide release during denitrification.

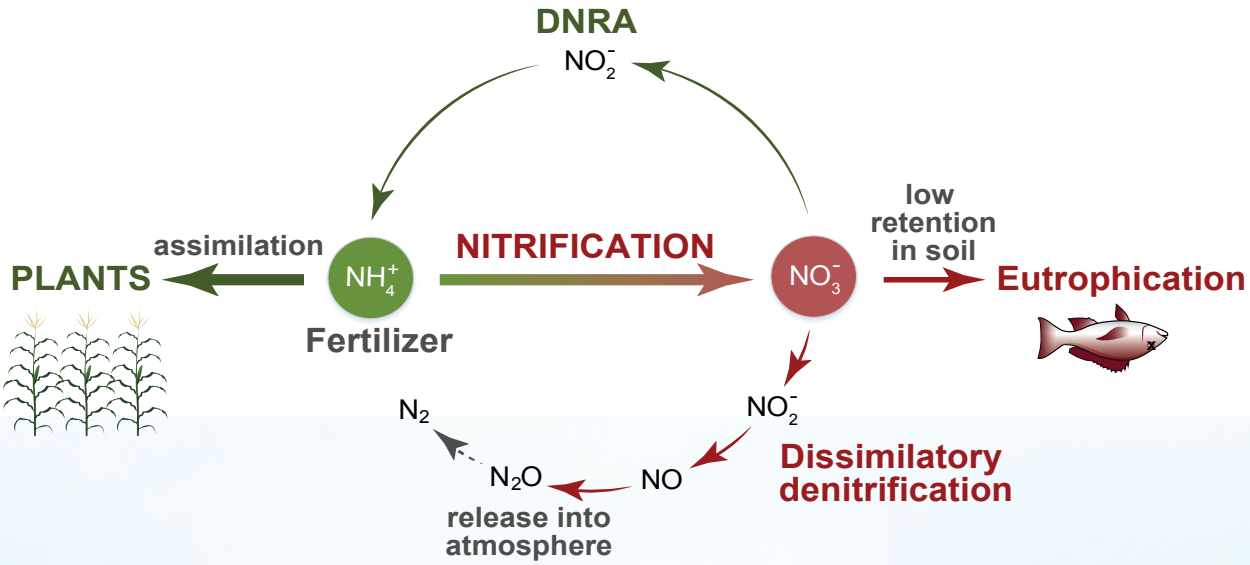


Figure 6. *Interaction of the nitrification, DNRA, and denitrification pathways.*

Research Approaches and Technology Needed

To stimulate the DNRA pathway ($NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$):

- Expand our understanding of the microbes performing this reaction, by probing the diversity of the microbial DNRA systems. Ultimately we need to ascertain the key players performing these reactions in agricultural soils. Metagenomics will be a useful tool in this effort.
- Determine the conditions that stimulate the growth of organisms performing the DNRA pathway in agricultural soils. One question to be answered is, can we stimulate the growth of these organisms by adding a small molecule to the soil, for example during fertilization of the agricultural soil?
- Identify methods to grow these organisms in pure culture, to aid in the study of this pathway in vivo. This will help determine the growth conditions under which the DNRA pathway in these organisms is upregulated. This approach involves determining what sensors, transcription factors, and proteins regulate the DNRA pathway, as well as the primary electron (i.e., carbon-based) sources for this pathway.
- Elucidate the molecular mechanism of cytochrome *c* nitrite reductase (ccNIR), which catalyzes the direct six-electron reduction of nitrite to ammonia, and engineer microbes to contain ccNIR and DNRA activity (Einsle et al., 1999).
- Develop a probe for a high-throughput ammonia-based activity assay to aid in the development of DNRA pathway screens.
- Develop a method for recycling water runoff from farms back onto fields after converting nitrate to ammonia.

To limit N_2O release from the denitrification pathway ($NO_3^- \rightarrow \rightarrow \rightarrow N_2$):

- Expand the phylogenetic diversity of the organisms and enzymes being studied, using microbiome analysis. What we know about denitrification today is based on detailed studies of relatively few organisms (Averill, 1996; Averill, 2007; Zumft, 1997). However, given the impressive biological diversity in nature, it is quite possible, and perhaps even likely, that there are differences in how other organisms reduce nitrogen compounds, in terms of the enzymes involved, efficiencies, the amount of N_2O lost, and other factors.
- Ascertain the primary (carbon-based) electron sources as well as the direct electron donors in the various steps of the pathway, using experimental

techniques as well as computer-assisted analysis of protein-protein and protein-small molecule interactions.

- Determine if and how the various enzymes in the pathway are coupled, both physically and metabolically.
- Extend our knowledge of the proposed yet elusive NO dismutase pathway, which would directly convert NO into N_2 and O_2, avoiding the generation of N_2O (Ettwig et al., 2010). Does this pathway exist in nature?
- Determine whether there are other physiologically relevant NO sinks.
- Determine the major microbial source of N_2O being released into the atmosphere under different growth conditions. Ratio analysis of isotopes (both as source tracers and as mechanistic probes) is an important technique for this goal.
- Ascertain what substrate or cellular resource is limiting in the $NO_3^- \rightarrow N_2$ pathway, and under what conditions. This will involve determining rate constants and concentrations of electron donors, O_2 sensitivity, and other factors; and determining whether the denitrification pathway can be stimulated by a soil additive.
- Measure the flux of nitrogen through the denitrification pathway under different growth conditions. This involves determining how important the abiotic reaction pathways are.
- Ascertain how denitrification is coupled to nitrification, and the chemical signals involved in the potential communication between the organisms involved in each pathway.
- Obtain a detailed understanding of the molecular mechanism of each of the enzymes in the pathway, to aid in the development of selective inhibitors. In particular, what is the mechanism of N-N bond formation and N-O bond cleavage in NO reductases, which are the critical enzymes responsible for N_2O formation? Other important enzymes to be studied include nitrous oxide reductases. This effort will require spectroscopic, kinetic, computational, and structural studies (e.g., crystallography and cryo-electron microscopy).
- Elucidate the missing details of the maturation pathways, i.e., the processes by which the active site in metalloenzymes are assembled, in, e.g., nitric oxide and nitrous oxide reductase biosynthesis. Such information could be useful in stimulating activity and/or engineering microbes.

- Genetically engineer nitrous oxide reductase into microbes lacking that enzyme, to facilitate enhanced N_2O reduction in the soil. Improved or engineered nitrous oxide reductase could also be used in this regard to optimize or improve the K_m, O_2 tolerance, etc., of these enzymes, in order to enhance the flux of N_2O being reduced to N_2.

To use plants to help maximize nitrate reduction through plant biotechnology:

- Engineer plants that stimulate the growth of DNRA microbes, by encouraging plant roots to secrete the preferred carbon source of DNRA microbes at certain times of the year, or via other engineering of preferred plant-microbe interactions.
- Develop cover crops that will concentrate NO_3^- and/or reduce it to organic nitrogen biomolecules, once the plant has taken up what it needs for optimal growth.

Expertise

Progress will require collaboration between chemists with a variety of subspecialties as well as experts from other fields. Within chemistry, expertise is required in biochemistry, bioinorganic chemistry, synthetic chemistry, bioanalytical chemistry, and isotopic biogeochemistry. Additional expertise is needed from molecular biology, enzymology, microbial ecology, microbial physiology, biophysics, crystallography, and structural biology.

Outcomes

The strategy outlined above will provide the research community with a much greater understanding of the specific organisms responsible for both the denitrification and the DNRA pathways, how these pathways are integrated into overall cellular physiology, and how these pathways can be down-regulated and/or stimulated. In addition, these studies will provide a detailed molecular mechanism for each of the critical steps in the respective pathways. Together, this information will allow the community to develop strategies to maximize nitrate reduction to either N_2 or NH_3 while simultaneously limiting the formation of or stimulating the reduction of N_2O. Ultimately these strategies can be used to minimize nitrogen loss from agricultural soils, mitigate the eutrophication of rivers and streams, and limit the release of the potent ozone-depleting greenhouse gas N_2O.

Improving how plants manage nitrogen

Nitrogen assimilation comprises the processes by which both oxidized (nitrate) or reduced (ammonia) nitrogen forms become linked to carbon-containing compounds that make up the bulk of all known life forms. Compounds containing both carbon and nitrogen

include amino acids, nucleic acids, proteins, chlorophyll, and a wide range of secondary metabolites. Such compounds form the organic nitrogen sources essential for life and biomass accumulation at all ecosystem levels. The nitrogen assimilation pathway consumes much of plants' energy, and is thus a key balance point in managing biological tradeoffs within and between organisms that drive the nitrogen cycle.

Because nitrogen-containing plant compounds are the basis of the human diet, workshop participants identified an urgent need for research into increasing how much nitrogen plants take up from soil. We determined that better answers to three important questions are essential to enhancing nitrogen assimilation.

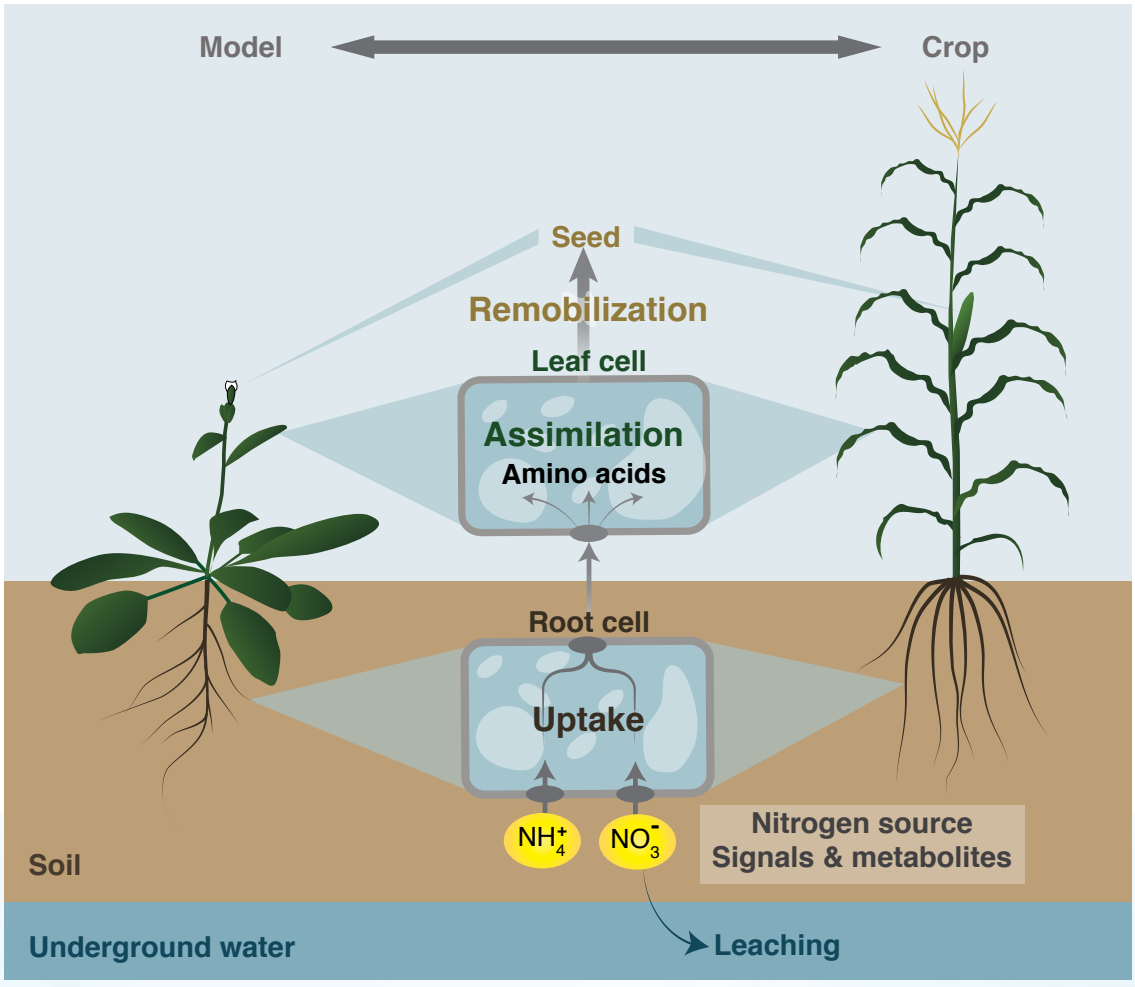


Figure 7. *Finding ways to increase plants' uptake of soil nitrogen compounds is critical to mitigating nitrogen's environmental impact and feeding the world.*

Question 1: What biochemical mechanisms are used by plant enzymes that catalyze nitrogen reduction, and that catalyze chemical bond formation between inorganic nitrogen and either carbon (amination) or sulfur (nitrosylation)?

Background

The initial reduction of nitrate to nitrite in plants is in some ways mechanistically similar to the first step of microbial denitrification, described above, so advances in understanding denitrification are also relevant to nitrogen assimilation. Scientists now recognize that nitrite (NO_2^-) is a key intermediate for interconversion among nitrogen forms, with similarities to molecules involved in the oxidation and reduction of carbon and sulfur (Maia and Moura, 2014).

Plants, however, achieve nitrate reduction to nitrite and then to ammonia using enzymes and pathways that are distinct from those of soil microbes, and coordinate the process with different organelles. Furthermore, plants can alter chemical reaction equilibria by moving substrates, products, or cofactors to different places within cells, or into and out of cells. Scientists also do not fully understand how electrons are transferred during these nitrogen interconversions under variable biological conditions.

Transport between organelles, between tissues, and across membrane systems at the interface between roots and soil also limits nitrogen assimilation in plants. Scientists do not fully understand how plants select and position membrane proteins that move different nitrogen forms into and out of cells. Similarly, scientists lack detailed knowledge of reaction mechanisms of many of the enzymes that catalyze the breakage and reformation of nitrogen-containing bonds in organic nitrogen compounds that play key roles in nitrogen transport, utilization, and remobilization. Examples include amino acids and ureides in nitrogen-fixing legumes.

Research Approaches and Technology Needed

- Create enhanced methods for an evolutionary genetics approach to identify functional variation among oxidation/reduction enzymes, and discover new enzymes and reaction mechanisms. This involves comparing variation in DNA sequences across organisms that also show differences in nitrogen assimilation, to reveal what sequence changes might contribute to the organisms' phenotypes. The power of this approach increases with the number of sequences compared, but the computational effort required is also greater.

- Analyze enzyme structures to guide protein engineering for greater catalysis, reduced sensitivity to inhibition, and faster transport. Develop methods to isolate, express, purify, and study membrane-localized nitrogen transport proteins.
- Develop methods for rapid testing of nitrogen transport activities, to better monitor and manipulate the dynamics of nitrogen metabolite flux within plants (in both model organisms and crop plants). Such methods could include spectroscopy, tomography, and imaging of both single-cell and whole-plant systems.

Expertise Needed

Progress will require collaboration between synthetic and bioanalytical chemists as well as among structural biologists and enzymologists, particularly those who study oxotransfer reactions catalyzed by metalloenzymes.

Outcomes

The research program described above will provide fundamental knowledge that will enable scientists to engineer key nitrogen transporters and assimilation enzymes to improve nitrogen uptake by plants from soil and assimilation into organic forms. It will provide detailed knowledge of nitrogen transport functions, which will inform strategies to reprogram and optimize nitrogen fluxes between organelles, cells, and organs; and ultimately to optimize plant growth.

Question 2: What chemical features of nitrogen signaling pathways regulate changes in nitrogen assimilation in plants?

Background

All living organisms possess nitrogen sensing and response systems, which are reasonably well understood in model microbes. Importantly, in multicellular plants, nitrogen compounds are both chemical metabolites and signaling molecules that promote a number of physiological responses, which are coordinated by small groups of regulatory genes (Gutiérrez, 2012; Krapp et al., 2014; Simons et al., 2014; Liu et al., 2015).

Indeed, nitrogen signaling and nitrogen metabolism have a long history of coevolution in nitrogen-limiting environments. As a result, plants have evolved to store some nitrogen rather than devote it all to growth, to ensure that they maintain a minimum reproductive

fitness even in harsh conditions. This evolutionary history does not promote high nitrogen use efficiency (NUE) in agricultural systems, where the goal is to maximize seed output no matter what the soil nitrogen supply is (Moose and Below, 2008).

Scientists have recently elucidated some of the biochemical details of nitrogen signaling pathways. Advances include identifying the first transceptor protein that functions to both transport nitrogen into and out of cells and induce physiological responses to sensed nitrogen (Gojon et al., 2014), recognizing that small signaling peptides play a role in nitrogen signaling, and identifying key hubs that coordinate the expression of many genes within transcriptional regulatory networks.

However, many important questions remain. Among the many inorganic and organic nitrogen forms with signaling roles, which are the most potent in programming desired physiological responses? How does sensitivity to nitrogen-related signals vary during plant development? How do the signals that plants and symbiotic nitrogen-fixing microbes exchange balance the tension between mutualism among partners and individual fitness, limit plants' choices in microbial partners, and impose sanctions on the allocation of plant carbon to poorly performing root nodules? At the level of agronomic productivity (Moose and Below, 2008), how much of the increase in plant growth response to nitrogen is due to sensing and acquisition of available nitrogen by roots; versus the capacity of vegetative tissues to assimilate, metabolize, and store nitrogen; versus nitrogen transport to new growth or seeds?

Research Approaches and Technology Needed

- Develop techniques for detailed genomic and physiological monitoring of how plant growth responds to nitrogen, the dynamics of nitrogen storage versus remobilization, and programmed senescence of plant tissues in both model organisms (e.g., *Arabidopsis*, *Setaria*, *Medicago*) and crops (e.g., maize, soybean).
- Investigate how nitrogen signaling inputs regulate the major photosynthesis pathways (C3, C4, and CAM).
- Design and synthesize non-metabolizable signal analogs that uncouple signaling from known roles in metabolism, or "suicide substrates" that block signaling, for use in screens to identify genes controlling nitrogen sensing.
- Develop strategies for delivering isotopic tracers (C, H, N) for ammonia or organic nitrogen, to reveal signaling pathways and metabolic fluxes within plants, among microbes, and between microbes and plants. This will require targeted use of

stable isotopes at the molecular level, and analytical tools to facilitate their measurement.

- Determine structure and function of signaling components through protein purification, kinetics, and structural studies. This will require methods to isolate, express, purify, and study potential nitrogen transporters and receptors.
- Evaluate the impact of mycorrhizal and Rhizobia symbionts on nitrogen signaling and response, and on nitrogen metabolism. This will require chemical or biological sensors of nitrogen signaling outputs.

Expertise Needed

To better understand the chemical features of nitrogen signaling in multicellular plants, we need interdisciplinary teams of chemists, microbial and plant biologists, agricultural scientists and engineers, and quantitative systems scientists.

Outcomes

The research program outlined above will lead to a better understanding of the mechanism of nitrogen signaling and its impact on nitrogen use efficiency on a molecular level. The mechanisms uncovered could help researchers modify signaling and/or nitrogen metabolism pathways to engineer or breed plants with increased NUE. Uncoupling the contribution of nitrogen signaling to nitrogen metabolism will create new opportunities to reprogram plant responses to nitrogen, possibly leading to designer microbes and plants (or selection of superior natural varieties) that better optimize crop growth with signal inputs. Other outcomes include alternative fertilizers with superior properties, including chemical stability, soil retention, and improved or more precise manipulation of signaling-response systems; and development of symbiotic microbe-plant systems that respond to plants' nitrogen needs, decreasing opportunity for waste.

Question 3: How can we promote environmentally resilient nitrogen use efficiency, especially in anticipation of changing environmental factors?

Background

Promoting environmentally resilient nitrogen use efficiency is an important goal for future food security. Plant NUE is naturally low—less than half of applied nitrogen is taken up by crops—and the chemical drivers for nitrogen availability and assimilation are themselves

affected by a variety of changing environmental factors, such as precipitation levels and air and soil temperature (Mueller et al., 2012).

Rising levels of greenhouse gases are predicted to increase temperatures; alter soil chemistry; and change the timing, frequency, and intensity of seasonal rainfall, causing both droughts and flooding.

Plants rely more heavily on uptake systems for organic nitrogen in cooler soils or in soil with low organic matter. Plant nitrogen signaling systems affect root architecture and hence a plant's abilities to tolerate drought and acquire nutrients, but we lack mechanistic understandings of how these interactions work. Indeed, we lack information about how the environmental conditions anticipated in the near future will change which nitrogen forms organisms assimilate. Most existing studies of plant nitrogen assimilation in cropping systems have been conducted in conditions of sufficient soil nitrogen and water.

Due to the interconnection between nitrogen and water availability, climate change could have particularly severe impacts on agricultural systems in which nitrogen is limited (Godfray et al., 2010). Because all the nitrogen that a plant takes up comes dissolved in water, drought reduces the nitrogen available for plants. Flooding can have similar effects, because it reduces soil oxygen, which reduces the activity of microbes that make nitrogen available to plants. Although scientists know that environmental conditions modulate the strength and amplitude of nitrogen signals (Bloom, 2014) and the expression of plant genes influencing NUE (Gutiérrez, 2012), we need more research into the biochemical outcomes and underlying mechanisms behind these environmental influences.

In addition, research has shown that rising levels of atmospheric CO_2 itself can inhibit plant nitrate assimilation and constrain the overall nitrogen cycle, shift the distributions of species within ecosystems, and interfere with CO_2 sequestration into biomass. Lower nitrogen assimilation could reduce the efficiency of photosynthesis, for example by causing plants to produce less chlorophyll or fewer nitrogen-rich enzymes. Elevated CO_2 often inhibits the biochemical reactions for rhizobial nitrogen fixation and nitrate assimilation, which may shift how heavily legumes depend on nitrate, ammonia, or organic nitrogen forms.

Research Approaches and Technology Needed

- Evaluate how abiotic stress conditions (drought, flooding, temperature extremes, high CO_2, levels of other nutrients) affect nitrogen assimilation and signaling, in both controlled and field environments.

- Investigate how other soil macronutrients (phosphorus, potassium) influence nitrogen signaling and overall nutrient use efficiency.
- Document the impacts of anticipated climate change on nitrogen assimilation processes within and across species (in both models and crops), microbial communities, and ecosystem levels.
- Develop rapid, efficient, and non-destructive phenotyping of plant NUE traits at population scales under variable environmental conditions.
- Refine climate-driven crop productivity models to account for recent and future genetic improvements and anticipated changes in nitrogen assimilation.

Expertise Needed

Promoting environmentally resilient nitrogen use efficiency will require interdisciplinary teams of chemists, microbial and plant biologists, agricultural scientists and engineers, and quantitative systems scientists. Modelers of climatic, ecological, and crop productivity will need to integrate the rapidly expanding datasets that are being generated by recent advances in the automated collection and processing of environmental and biological properties. Such "big data" offers novel opportunities to better estimate location-specific impacts of variability in nitrogen sources to net primary plant productivity.

Outcomes

The approaches outlined above will help make plant NUE resilient to expected environmental variability in both the developed and developing world, in order to meet future food demands. They will also help us better understand the balance between economic and environmental costs of nitrogen inputs.

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Appendix 1: Workshop participants

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Appendix 2: Workshop agenda

Day 1: Monday, Nov. 9

TIME	TASK
8:00 – 9:30 am	Registration and breakfast; explanation of the structure of the workshop
9:30 – 10:15 am	Plenary Lecture 1: Rob Horsch (Bill and Melinda Gates Foundation), "Agricultural Alchemy" DISCUSSION
10:30 – 10:45 am	Coffee Break
10:45 – 11:30 am	Plenary Lecture 2: Mark David (UIUC), "The Agricultural Nitrogen Cycle: Why is it so Difficult to Maximize Production and Reduce Environmental Impacts?" DISCUSSION
11:45 am – 1:00 pm	Working lunch: Introduction of the four focus areas by the session chairs
1:00 – 1:30 pm	Coffee Break
1:30 pm – 2:45 pm	Breakout Session 1 • Introduction of participants (5-minute presentation/ speaker) • Session chairs and/or scribes take note of all proposed BIG questions • First round of discussion: where is consensus, where is controversy?

2:45 pm – 3:15 pm	Coffee Break. Participants score the BIG questions in their corresponding sessions. • Session chairs & scribes write down the most important questions from the scoring process
3:15 pm – 4:30 pm	Breakout Session 1 (cont.): Imagine BLUE SKY solutions to solve these important questions! Go question by question, in order of ranking. • What expertise is needed to solve/overcome the problem? • What technology needs to be developed? • What basic knowledge needs to be acquired? • What is the desired outcome?
4:30 pm – 5:00 pm	Coffee Break. Session chairs and scribes prepare summary documents.
6:15 pm	Dinner and Summary Session 1: All participants meet @ Rustico for dinner. Session chairs summarize main findings from their breakout session.
Evening	Organizers create new breakout sessions around the four most important scientific questions/problems of the nitrogen cycle.

Day 2: Tuesday, Nov. 10

TIME	TASK
8:00 – 9:15 am	Breakfast: introduction of the four new breakout session topics, organizational remarks
9:30 – 10:30 am	Breakout Session 2: Session chairs provide a summary of the discussion on the topic and introduce conflicting approaches/thoughts/angles to start the discussion. • NEXT: Imagine BLUE SKY solutions to solve these important questions! » What expertise is needed? » What technology or knowledge? » What experiments need to be conducted? » What is the best possible solution? » How do we get there?
10:30 – 11:00 am	Coffee Break
11:00 – 12:30 pm	Breakout Session 2 (cont.): Participants make a research plan (outline style, plan of action) of how to tackle their BIG question. The more concrete the better!
12:30 – 2:00 pm	Working lunch: Session chairs and scribes prepare summary documents; further discussion of the four BIG questions.
2:00 – 4:00 pm	Session chairs present their summaries to all participants. Final discussion about the questions, and how they are interrelated.
4:00 – 6:00 pm	Organizers, science writer prepare the final (short) summary document for NSF.

FEEDING THE WORLD IN THE 21ST CENTURY: GRAND CHALLENGES IN THE NITROGEN CYCLE

NATIONAL SCIENCE FOUNDATION

Arlington, VA | November 9–10, 2015

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